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Original Investigation

Neuroprotective Effects of Chronic Fenofibrate Treatment via Modulating the Immunoreactivity of Cleaved Caspase-3 in Stroke Induced by Transient Middle Cerebral Artery Occlusion Rat Model

Ozge ALTINTAS¹, Mehmet Ozgen ALTINTAS², Mehmet Serif AYDIN³, Oguz BARAN⁴, Veysel ANTAR⁴, Mukaddes ESREFOGLU³, Talip ASIL⁵

¹Bor State Hospital, Neurology Clinic, Nigde, Turkey

²Fatih University, Faculty of Engineering, Department of Genetics and Bioengineering, Istanbul, Turkey

³Bezmialem Vakif University, Department of Histology and Embriology, Istanbul, Turkey

⁴Istanbul Research and Training Hospital, Neurosurgery Clinic, Istanbul, Turkey

⁵Bezmialem Vakif University, Department of Neurology, Istanbul, Turkey

ABSTRACT

AIM: Current stroke therapies include lipid-lowering drugs, which reduce inflammation and serve to stabilize the atherosclerotic plaque to demonstrate better outcome and neuroprotection. Peroxisome proliferator activated receptors (PPAR) α regulates lipid homeostasis and is a target of fibrates, which have a neuroprotective function by various mechanisms. In this study, we aimed to evaluate the role of the PPAR α agonist, fenofibrate, in the modulation of cleaved caspase-3 immunoreactivity and at the final infarct volume in an experimental ischemia/reperfusion rat model by induced transient proximal middle cerebral artery occlusion.

MATERIAL and METHODS: A total of 65 male Sprague Dawley rats were allocated into 4 groups; sham (n=5), experiment 1 (n=20), experiment 2 (n=20), experiment 3 (n=20). All experiment groups were divided to 3 subgroups in order to evaluate the final infarct volume at 24th hour (n=5) and the immunoreactivity of cleaved caspase -3 at different time periods [at first hour (n=5), at 6th hour (n=5), at 24th hour (n=5)] after transient middle cerebral artery occlusion (MCAo). At the study, the experiment groups (Experiment 1 and Experiment 2) were received the fenofibrate-diet during 14 days before ischemia procedure. All animals were sacrificed at 24th hours after MCAo. Infarction volumes were calculated from 2,3,5,triphenyltetrazolium chloride (TTC)- stained brain sections.

RESULTS: We found that fenofibrate-therapy reduced significantly more body weight than the other experiment groups ($p<0.05$). At the time intervals, a decrease of immunoreactivity of cleaved caspase-3 was significantly observed with fenofibrate therapy after MCAo ($p<0.05$). Chronic fenofibrate treatment before cerebral ischemia significantly reduced the infarction size after MCAo compared with the other groups (respectively; $p = 0.011$ and $p < 0.000$).

CONCLUSION: Fenofibrate treatment has neuroprotective effects on middle cerebral artery infarcts.

KEYWORDS: Neuroprotection, Fenofibrate, Stroke, Caspase-3



Corresponding author: Veysel ANTAR

E-mail: veyselantar@hotmail.com

■ INTRODUCTION

Stroke is the second leading cause of mortality and the third leading cause of morbidity worldwide (16). Current therapies for ischemic stroke include primary and secondary preventive therapies. Particularly, these new stroke prevention therapies may help to reduce inflammation, serve to stabilize the atherosclerotic plaque, or act by other protective mechanisms (10). Lipid lowering drugs also exert a preventive neuroprotection in strokes that occur despite the preventive measures, as demonstrated by a better outcome for patients receiving lipid-lowering drugs prior to stroke occurrence (5). The basic mechanisms involved in this preventive neuroprotection have been identified in experimental models. For fibrates, the mechanism involves the nuclear receptor, peroxisome proliferator activated receptors alpha (PPAR α) (7).

Peroxisome proliferator activated receptors (PPARs), members of the nuclear receptor superfamily, are ligand-activated transcription factors with diverse actions including regulation of adipocyte differentiation and lipid metabolism (22). PPAR α regulates lipid homeostasis and is a target of fibrates, which are used clinically for the treatment of hypertriglyceridemia (20). The PPAR α agonist fenofibrate has a neuroprotective function by inhibiting the central mediator, Tumor necrosis factor (TNF), of neuroinflammation and apoptosis due to antagonize activities of transcription factors such as NF- κ B (2,3). TNF is cytokine, which is the major extrinsic mediator of apoptosis (8).

Apoptotic cell death is a highly regulated process that in many cases requires activation of caspases. Caspase-3, an executioner caspase, plays a central role in apoptosis following transient brain ischemia (14). Studies have suggested that caspase-3 activation does not mean the cells are dead. Cleavage of caspase-3 is therefore a well-established marker for apoptotic cell death (18).

In this study, we aimed to evaluate the role of the PPAR α agonist, fenofibrate, in the modulation of cleaved caspase-3 immunoreactivity and the final infarct volume in an experimental ischemia/reperfusion rat model by induced transient proximal middle cerebral artery occlusion.

■ MATERIAL and METHODS

Animals

All animals were obtained from the Experimental Animal Research Laboratory at Bezmialem Vakif University, Istanbul, Turkey. Animals were allowed free access to food and water at controlled room temperature (22–25°C) under a 12:12-h day/night cycle for the duration of the study. During the surgical procedures, body temperature was monitored using a Nimomed infrared thermometer. All procedures were approved by the Animal Care and Use Committee at Bezmialem Vakif University and performed in accordance with institutional guidelines.

Middle Cerebral Artery Occlusion (MCAo)

The most common stroke model, due to its relevance to human

stroke, is focal MCAo (11). In the present study, we induced transient proximal MCAo to cause ischemia-reperfusion injury to assess the neuroprotective effect of prophylactic chronic treatment with fenofibrate. Animals underwent a 1-hour MCAo, followed by reperfusion for 24-hours.

Moreover, studies have found that cleaved caspase-3 activity increased gradually within 24 hours in the infarct core after transient MCAo; but the activity was overall lower in the nonperfused core after permanent occlusion, suggesting that caspase-3 activity depends on reperfusion by the fact that free radicals generated during reperfusion (6,14,18). So at our study, we demonstrated the ischemia-reperfusion MCAo rat model to observe the cleaved caspase-3 association with ischemic infarct volume.

Focal cerebral ischemia was induced using an endovascular middle cerebral arterial occlusion technique, as described previously (11). Briefly, animals were anesthetized with ketamine (4 mg/100 g) and xylazine (1.5 mg/100 g) by intramuscular injection and placed on an operation plate in the supine position. Their heads and limbs were fixed. After shaving and sterilization, a cervical median incision (3–4 cm long) was made. Precervical fascia and muscle were isolated with forceps, and fascia and muscle on the inside of the sternocleidomastoid were dissociated. Arterial pulses were visible. Tissues surrounding the artery were carefully dissociated, without injury to the vagus nerve. The left common carotid artery and the left external carotid artery were exposed through a midline neck incision. The proximal part of the left common carotid artery and root of the external carotid artery were ligated. The pterygopalatine artery was dissociated upwards along the internal carotid artery, and ligated. A 4.0 Medium B MCAO monofilament nylon suture (Doccol), whose tip had been coated with silicone, was then inserted through an arteriotomy of the left common carotid artery and gently advanced into the internal carotid artery to a point ~15–16 mm distal to the carotid bifurcation. We used Gerriets and colleagues' method (9) to assess the success of the MCA occlusion. Therefore, the method is not useful to document that the MCA occlusions are uniform. Mild resistance to this advancement indicated that the suture had entered the anterior cerebral artery, thus occluding the origins of the MCA. The left common carotid artery was loosely ligated just distal to the arteriotomy, after which the neck wound was closed. After 60 minutes, the suture was carefully removed; its tip was blocked by a microclamp placed on left common carotid artery to allow reperfusion. The sham operation consisted of the same manipulation but without introduction of the monofilament.

Study Design

Sixty-five male Sprague-Dawley rats (450–500 g; 10–12 months old) were allocated to four groups; sham operated group (n=5), experiment-1 group (n=20), experiment -2 group (n=20), and experiment-3 group (n=20). Transient proximal MCAo was induced in all experiment groups after treated drugs during 14 days. All experiment groups were divided to 3 subgroups in order to evaluate the final infarct volume at 24th hour (n=5) and the immunoreactivity of cleaved caspase -3 at different time period [at first hour (n=5), at 6th hour (n=5), at

24th hour (n=5) after MCAo. All animals were sacrificed at 24th hours after MCAo. Also all animals were weighed everyday during the study period. Study design was showed at Table I.

Drug Administration

The experiment groups (Experiment-1 and Experiment-2) received the fenofibrate diet for 14 days before ischemia procedure, because mechanisms of fibrate-induced preventive neuroprotection involve genomic regulation by nuclear receptor PPAR α pathways.

A-Sham operated Group (n=5): These animals were fed a standard diet. This group did not undergo any surgical procedure.

B-Experiment-1 Group (n=20): These animals were treated for 14 days with vehicle (10% (v/v) Dimethylsulfoxide (DMSO) 2 ml/day) by oral administration through a feeding needle. Dimethylsulfoxide (DMSO) is a common vehicle used for many drugs used in neuroprotective experiments. DMSO has many biological effects, including anti-inflammatory, antioxidant, and local anesthetic effects and recently the researches investigated its neuroprotective effects (23). So we decided to allocate the vehicle group to the study due to determine the neuroprotective effect of fenofibrate. This group was called DMSO+MCAo.

C-Experiment-2 Group (n=20): These animals were treated for 14 days with Fenofibrate (FB) (200 mg/kg was dissolved in 10% (v/v) Dimetil Sulfoxide (DMSO) 2ml/day) by oral

Table I: The Study Groups, Procedures and Number of Rats in Each Group

Study Groups	Procedures	Number of rats in each group
Sham-operated	This group did not undergo any ischemic or surgical procedures	5
	Assessment of immunoreactivity of cleaved caspase-3	5
Experiment-1 (DMSO+MCAo)	Left pMCAo was done after treated with vehicle (DMSO)	20
	1- Assessment of immunoreactivity of cleaved caspase-3 at first hour after left pMCAo	5
	2- Assessment of immunoreactivity of cleaved caspase-3 at 6 th hour after left pMCAo	5
	3- Assessment of immunoreactivity of cleaved caspase-3 at 24 th hour after left pMCAo	5
	4- Assessment of infarct volume at 24 th hour after left pMCAo	5
Experiment- 2 (FB+DMSO+MCAo)	Left pMCAo was done after treated with Fenofibrate	20
	1- Assessment of immunoreactivity of cleaved caspase-3 at first hour after left pMCAo	5
	2- Assessment of immunoreactivity of cleaved caspase-3 at 6 th hour after left pMCAo	5
	3- assessment of immunoreactivity of cleaved caspase-3 at 24 th hour after left pMCAo	5
	3- Assessment of immunoreactivity of cleaved caspase-3 at 24 th hour after left pMCAo	5
Experiment-3 (MCAo)	Left pMCAo was done after feeding with a standard diet	20
	1- Assessment of immunoreactivity of cleaved caspase-3 at first hour after left pMCAo	5
	2- Assessment of immunoreactivity of cleaved caspase-3 at 6 th hour after left pMCAo	5
	3- Assessment of immunoreactivity of cleaved caspase-3 at 24 th hour after left pMCAo	5
	4- Assessment of infarct volume at 24 th hour after left pMCAo	5

administration through a feeding needle. This group was called FB+DMSO+MCAo.

D-Experiment-3 Group (n=20): These animals were fed a standard diet during 14 days. This group was called MCAo.

Assessment of Infarct Volume

Infarct volumes were calculated using 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain sections, as described previously (1). Briefly, after the animals were sacrificed, the brains were removed immediately and cut into 2-mm sections. Samples were then incubated for 30 min in a 2% solution of TTC at 37°C and fixed by immersion in 10% buffered formalin solution. Five brain sections per animal were stained with TTC and then photographed. Cerebral infarct volumes were calculated using the image analysis program of Adobe Photoshop CS5 extended (version 12.1).

Assessment of Immunoreactivity of Cleaved Caspase-3:

10 µm coronal sections of brain were cut with cryostat (CM1520, Leica Bio systems Nussloch GmbH) and were fixed with 4% paraformaldehyde for 1h. Caspase 3 activity was determined in coronal cryosections of ischemic hemispheres using the rabbit polyclonal anti-active + pro Caspase 3 primary antibody (1:100, Cat. # ab13847, Abcam, UK), which had been incubated for 18h at 4°C followed by repeated washing steps. Thereafter, goat anti-rabbit alexa fluor 488 secondary antibody (1:400, Cat. # A-11034, Thermo Fisher Scientific) was used for 1h at room temperature. Sections were counterstained with propidium iodide (500nM, Cat. # P3566, Thermo Fisher Scientific). Stainings were analyzed by quantifying caspase-3 positive cells in twelve ROI under a confocal microscope (LSM 780, Carl Zeiss, Jena, Germany).

Statistical Analysis

Continuous measurements are expressed as mean and standard deviation for each group. ANOVA and Kruskal-Wallis tests were used to assess the effectiveness of intervention and group differences in infarct size and weights of rats, along with levels of caspase-3. Dunn's test (non-parametric) and Tukey (parametric) test was used for multiple comparisons after Kruskal-Wallis and ANOVA, respectively.

P-values < 0.05 were considered statistically significant.

■ RESULTS

Mortality

None of animals died during the study period.

Effect of Fenofibrate on Body Weight

Since fenofibrate seems to act as a weight-stabilizer on genetic/diet-induced obesity mainly through its effect on liver metabolism (15), we decided to observe its metabolic effect on the body weight of the rats every day.

Before MCAo, at the 7th day and 14th day, the study groups that were treated with DMSO +FB or only DMSO lost weights significantly compared to other groups that were fed a standard diet (at 7th day FB+DMSO+MCAo vs. MCAo

p=0.016; DMSO+MCAo vs. MCAo p=0.010 and at 14th day FB+DMSO+MCAo vs. MCAo p=0.005; DMSO+MCAo vs. MCAo p=0.003, respectively). Moreover, we investigated that fenofibrate-therapy significantly reduced body weight rather than vehicle-therapy group (at 7th day and at 14th day, FB+DMSO+MCAo vs. DMSO+MCAo p<0.000). The body-weights of the study groups were showed at Figure 1.

These results suggest that changes in body weight were associated with the therapeutic potential of Fenofibrate.

Time Course of Cleaved Caspase-3 Immunoreactivity in Ischemic Brain

The immunoreactivity of positive caspase-3 cells was detected at the first hour, 6th hour and 24th hour after left MCAo. Among the study groups, the caspase-3 cleavage activation was not significantly different at the first hour after MCAo (p>0.99 among groups) (Figure 2). At the 6th hour and at 24th hour after MCAo, Caspase -3 cleavage activity was significantly lower in the FB+DMSO+MCAo group relative to the DMSO+MCAo and MCAo groups (p=0.005 and p=0.001 at the 6th hour; p=0.003 and p=0.004 at the 24th hour after MCAo, respectively), (Figure 2). Therefore; at these intervals, a significant decrease in the immunoreactivity of cleaved caspase-3 was not observed with DMSO (vehicle) therapy after MCAo (p>0.99) (Figure 2). The temporospatial distribution of cells with cleaved caspase-3 after transient MCAo is shown in Figure 3.

Collectively, these data demonstrate that chronic fenofibrate treatment as a PPAR activator could protects the neurons against ischemia by modulating the early phase of apoptosis. Moreover, we also suggest that this neuroprotective effect is independent of its vehicle.

Fenofibrate pre-treatment reduces the total infarct volume

TTC-derived infarct volumes (mm³) in the FB+DMSO+MCAo, DMSO+MCAo and MCAo groups are shown in Figure 4. Chronic fenofibrate treatment before cerebral ischemia significantly reduced infarction size after MCAO compared with the other groups (respectively; p =0.011 and p< 0.000). A TTC-stained brain section is shown in Figure 5A-C.

■ DISCUSSION

In the present study, we have demonstrated (in the rat) that a 14-day pre-ischemic administration of fenofibrate significantly decreases the cerebral infarct volume. This neuroprotective effect was associated with partial prevention of apoptosis after ischemia-reperfusion injury.

The mechanism by which PPARα activation by fenofibrate exerts cerebrovascular protective effects remains unknown. The neuroprotective effect of fenofibrate is independent of the well-documented lipid lowering effects of PPARα activators reported in various studies. At the Cochrane database, the researches have recently suggested that the fibrate class can be effective in the secondary prevention of composite outcome of non-fatal stroke, non-fatal MI, and vascular death (25). The normalization of the hypertriglyceridemia and hyperglycaemia by fenofibrate might contribute to its neuroprotective effect by

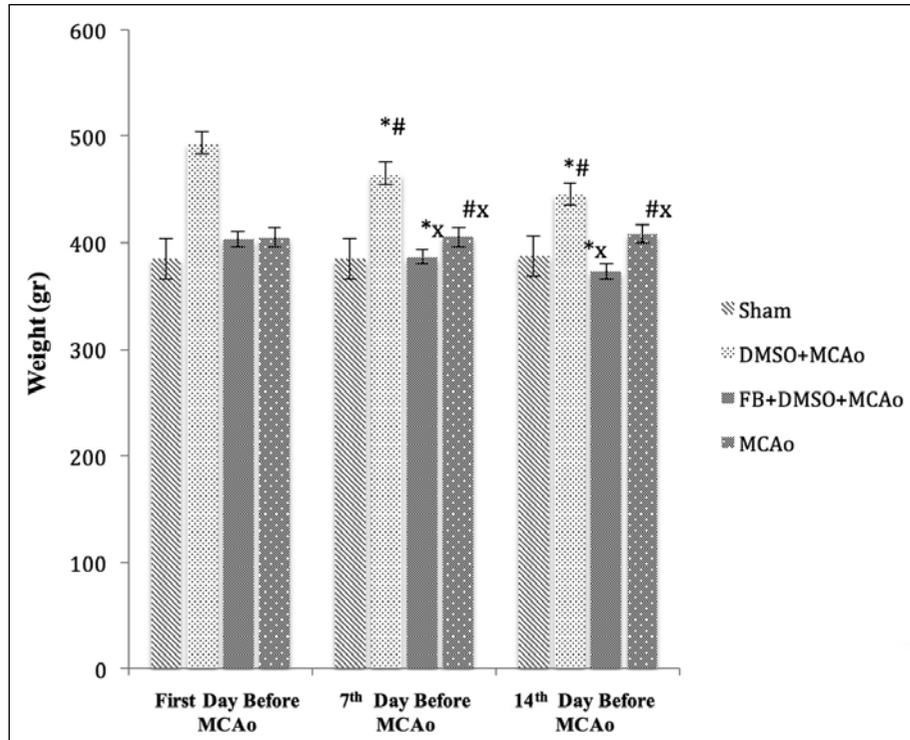


Figure 1: Rats were weighted during 14-day before MCAo. The data are expressed as mean ± standard error (*# p<0.05, Kruskal–Wallis test, post hoc Dunn’s Test). [For each group respectively; First day, Sham=385±19.20 gr; DMSO+MCAo= 493.45±10.71 gr; FB+DMSO+MCAo= 403.45±7.09 gr; MCAo= 404.70±9.05 gr 7th day, Sham=385±19.20 gr; DMSO+MCAo= 465±8.93 gr; FB+DMSO+MCAo= 386.90±5.72gr; MCAo= 405.20±9.40 gr 14th day, Sham=387.80±19.66 gr; DMSO+MCAo=445.65±5.82 gr; FB+DMSO+MCAo=373.30±5.49gr; MCAo= 408.40±8.98 gr].

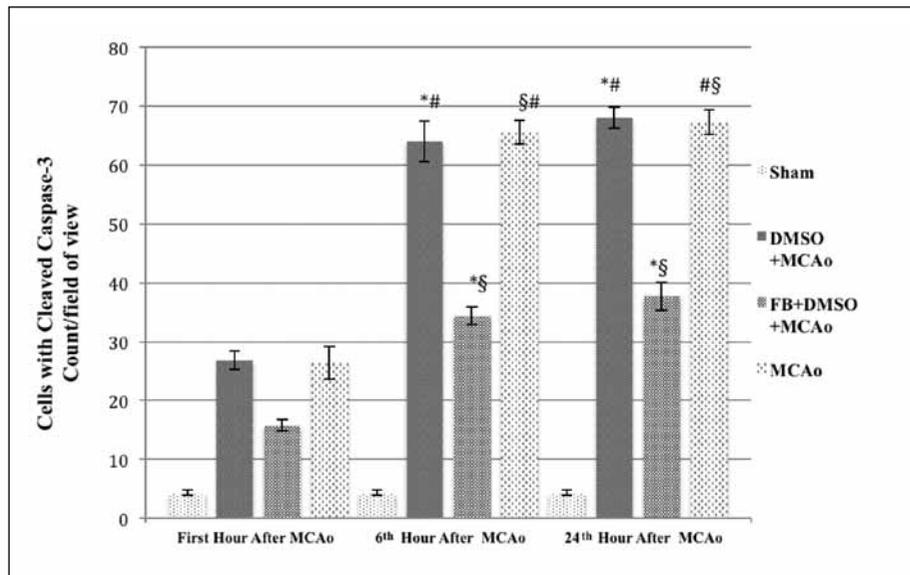


Figure 2: Quantitative analysis of cells with cleaved caspase-3 (count/ field of view) at first, at 6th and 24th after MCAo were established under a confocal microscope (LSM 780, Carl Zeiss, Jena, Germany). The data are expressed as mean ± standard error (*# p<0.05, Kruskal–Wallis test, post hoc Dunn’s test). For each group respectively; Sham = FB+DMSO+MCAo= 14.26±4.05 mm³; DMSO+MCAo= 34.58±2.07mm³; MCAo= 60.80±8.71 mm³ (p >0.99) (*, p=0.005; x, p=0.001; #, p>0.99) (*, p=0.003; x, p=0.004; #, p>0.99).

decreasing anti-atherotrombogenic factors (12). However, in normoglycemic or normolipidemic experimental rat ischemic models, studies also showed the neuroprotective effect of fenofibrate (4). We therefore evaluated the molecular target activated by fenofibrate to prevent the brain after ischemia.

During acute neuronal damage, necrosis is the most prominent cell death phenotype in the infarct core, whereas apoptosis predominates at the penumbra. Previous data showed that the post-ischemic activation of NF-κβ dependent genes has a key modulation on proinflammatory and proapoptotic

mediators. Activation of PPARα by binding of fenofibrate leads to inhibition of NF-κβ and concurrently the inhibition of apoptosis (21,24). Caspase-3, which is considered the prototype of executioner caspases and is the most plentiful caspase in the brain dependent apoptosis leads to potentially important implications for developing therapeutic strategies especially for stroke (8). At our study, we evaluated time-dependent changes in cleaved caspase-3 during reperfusion after transient MCAo. Cleaved caspase-3 did not appear in the normal brain; but the immunoreactivity changed over time when measured in three different time points after MCAo. The

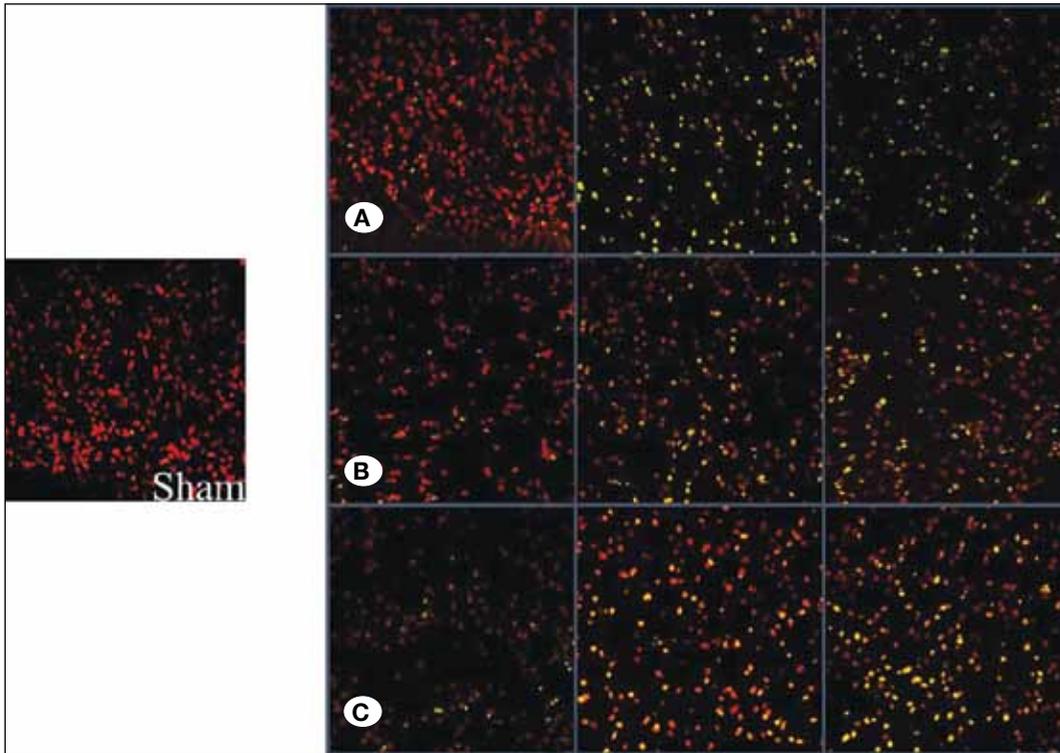


Figure 3: Pre-ischemic Fenofibrate administration inhibits the expression of cleaved caspase-3 at first hour, at 6th hour and 24th hour after transient MCAo. Stainings were analyzed by quantifying caspase-3 positive cells (green) in brain tissue specimens in twelve ROI under a confocal microscope (LSM 780, Carl Zeiss, Jena, Germany). **A)** DMSO+MCAo group at first, at 6th hour and 24th hour after transient MCAo. **B)** FB+DMSO+MCAo group at first hour, at 6th hour and 24th hour after transient MCAo. **C)** MCAo group at first hour, at 6th hour and 24th hour after transient MCAo.

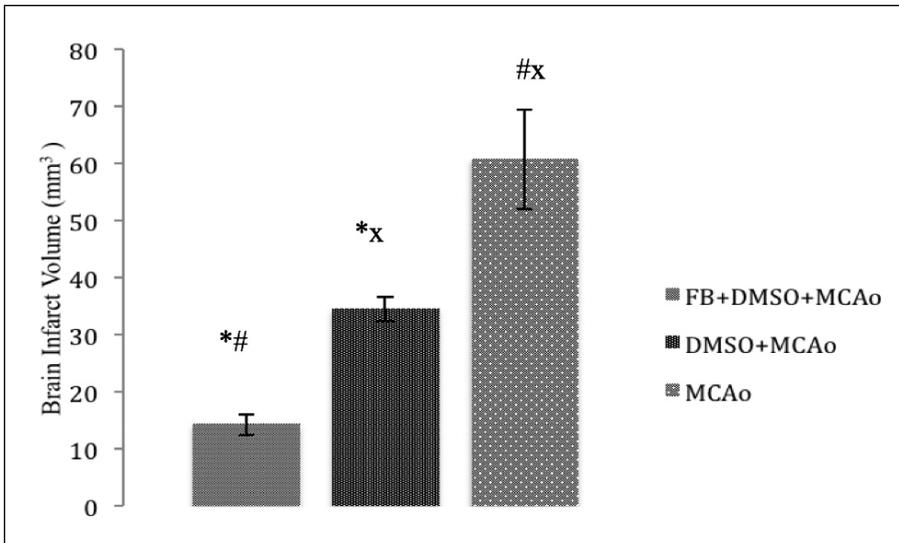


Figure 4: Brain infarct volume (mm³) at 24th hour after MCAo. The data are expressed as mean ± standard error (*# p<0.05, Kruskal –Wallis test). For each group respectively; FB+DMSO+MCAo= 14.26±4.05 mm³; DMSO+MCAo= 34.58±2.07mm³; MCAo= 60.80±8.71 mm³.

level immunoreactivity of cleaved caspase-3 was decreased significantly in the pre-ischemic fenofibrate treatment group.

Our findings are in good agreement with other studies having demonstrated the neuroprotective effect of modulating the nuclear PPARα receptors. In a recent study, the importance of the anti-inflammatory effect had already been suggested to prevent the decrease of vascular adhesion protein expression in the ischemic zone (19). Deplanque et al. showed that chronic treatment with fenofibrate increased major antioxidant enzymes including copper/zinc superoxide dismutase in the nonischemic mouse brain, which is likely to contribute to the neuroprotection in the brain after ischemia (4). Another

study showed that pretreatment with fenofibrate improved the penumbral cerebral blood flow during MCAo (17). Recently, Losey et al. observed that a 14-day fenofibrate pre-treatment decreased reactant production, infarct volume, and neutrophil recruitment to the brain and liver, which is a hallmark of the acute phase response (14).

CONCLUSION

Pre-ischemic administration of fenofibrate has a preventive protective effect against brain ischemia by decreasing infarct volume related to possible genomic modulation in

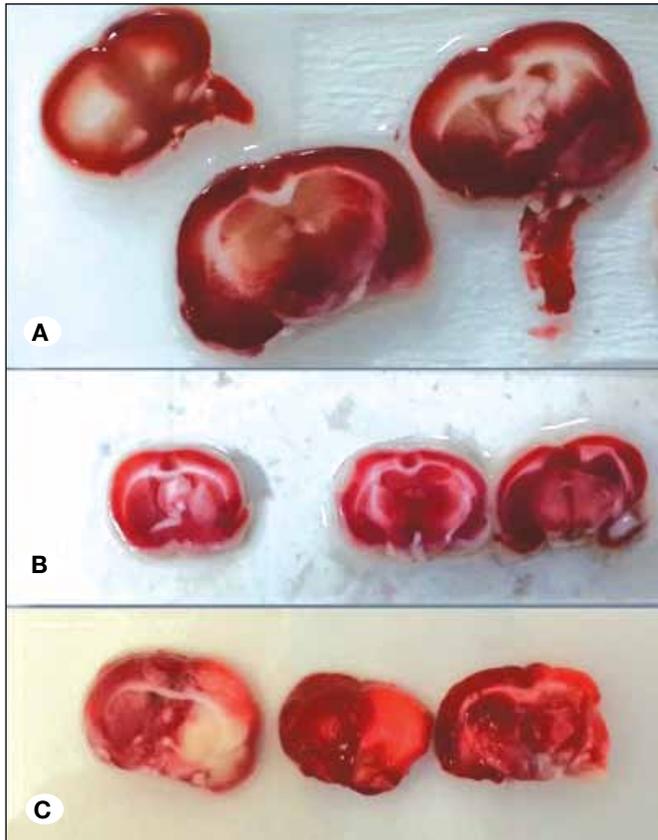


Figure 5: Brain sections were stained with TTC, demonstrating ischemic injury. Ischemic infarcts (white areas indicating brain damage) are substantially smaller in FB+DMSO+MCAo group.

A) DMSO+MCAo group, **B)** FB+DMSO+MCAo group, **C)** MCAo group 24 hours after transient MCAo.

the apoptosis pathway. Moreover, further information on the ability to prevent injury by inhibiting signaling cascades upstream of caspase-3 activation should reveal the functional role of apoptosis inhibition for treatment of stroke.

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